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Distribution of Fibronectin During Wound Healing *in Vivo*

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The distribution of fibronectin during wound healing has been studied. Full thickness wounds were made in the skin on the sides of guinea pigs' trunks. Biopsy specimens were taken from the normal skin, from the 5-hr-old wound, and on days 1–18 after wounding. Unfixed frozen sections were analyzed for fibronectin distribution by indirect immunofluorescence analysis with a specific antiserum prepared in rabbits against guinea pig plasma fibronectin. Tissue samples were also embedded in methacrylate and sections cut and stained with hematoxylin and eosin for general histology or with a silver stain for reticulin. Fibronectin was prominent in the basement membranes of normal skin epidermis. It was also present in the papillary dermis and to a lesser extent in the reticular dermis. After wounding, fibronectin was part of the fibrin clot and distributed along fibrin strands. Fibronectin was also deposited along newly synthesized collagen in the granulation tissue, which was at least in part collagen type III based upon staining for reticulin. Eventually, the entire granulation tissue was transformed into aligned collagen fibrils coated with fibronectin. Throughout the period of wound healing, the level of fibronectin associated with what appeared to be type I collagen in the reticular dermis adjacent to the wound area stayed about the same. When fibrils with the histological characteristics of type I collagen were within the granulation tissue, however, they were coated with fibronectin. The results indicate that fibronectin is a major component present during wound healing.

Fibronectins are a group of glycoproteins found in plasma (cold insoluble globulin) and secreted by a variety of cell types including fibroblasts, endothelial cells, and hepatocytes [1,2]. *In vitro,* the fibronectins have been shown to play an important role in cell adhesion to biological and material surfaces [1–3]. Nevertheless, the biological significance of fibronectin *in vivo* is unknown.

During clotting of plasma, fibronectin becomes associated with the clot [4], covalently crosslinking to fibrin through the action of factor XIII (fibrin stabilizing factor) [5,6]. Adhesion of fibroblasts to fibrin requires the presence of fibronectin and is maximal if the fibronectin is crosslinked to the fibrin [7]. Moreover, fibroblasts do not grow in fibrin clots prepared from Factor XIII deficient plasma [8,9] and patients with Factor XIII deficiencies display impairment of wound healing [10,11]. Fibronectin also promotes phagocytosis of particles, not only by macrophages [12,13], but also, by fibroblasts [14]. Finally, the deposition of collagen by fibroblasts can occur concomitantly with that of fibronectin [15–17]. These observations indicate that fibronectin may have profound effects on wound healing, including formation of the proper substratum for migration and growth of cells during the development and organization of granulation tissue as well as during remodeling and resynthesis of the connective tissue matrix.

Various studies carried out *in vivo* support the idea that fibronectin may play a role in wound healing. Plasma fibronectin levels are decreased in acute inflammation [18] or following surgical trauma [19] and in patients with intravascular disseminanted coagulation [20]. Moreover, fibronectin is located predominantly in the basement membranes of adult tissues [21–22] but may be more widely distributed in inflammatory lesions [22–25]. Since a systematic analysis of the tissue distribution of fibronectin during wound healing had not been carried out we studied full thickness cutaneous wounds that were prepared on the trunks of guinea pigs [26] and determined the appearance and distribution of fibronectin during the initial inflammatory response and during the subsequent development and organization of the granulation tissue. An account of this work forms the subject matter of this communication.

MATERIALS AND METHODS

Procedure for Making Wounds

Experimental animals were domestically bred strain #13 guinea pigs of either sex weighing approximately 750 gm. Anesthesia was induced by intraperitoneal injection of sodium pentobarbitol (50 mg/ml diluted 1:5 with sterile saline), 0.15 ml/100 gm body weight, followed by intramuscular injection of Innovar-Vet, 0.06 ml/100 gm body weight. The skin of the right side of the animal's trunk was shaved and painted with Wescodyne, an iodine based antiseptic. Rectangular wounds were made in the skin overlaying the rib cage of the right thoracic wall. They were approximately 4 cm × 6 cm in outline and to the depth of the panniculus carnosus muscle. Full details concerning the preparation of these wounds and the dressings applied have been presented elsewhere [26]. Suffice it to say that Furacin powder was applied to all unepithelialized wounds and that the immediate dressing was fine-meshed tulle gras.

Biopsy of Specimens

Specimens were taken at regularly spaced intervals from intact skin or wounds of etherized animals. Apart from normal skin taken at the time of surgery, specimens approximately 0.75 cm × 0.5 cm were taken down to the level of the muscle. The wounds were then redressed.

The samples were immediately sliced into 1-mm wide strips with the aid of a stainless steel single-edged razor blade (Weck Prep Blade) and subsequently either fixed in 4% formaldehyde in 0.1 M Na cacodylate buffer (pH 7.4) overnight at 4° or placed into appropriately sized embedding containers (e.g., Beem capsules) filled with embedding medium for frozen tissue specimens (O.C.T. Compound) and immersed in Dry Ice/acetone. Throughout the procedures, care was taken to maintain the specimens in a known orientation.

Light Microscopy

Tissue specimens were dehydrated through a graded series of ethanol solutions and finally infiltrated with JB-4 methacrylate (activated with benzoyl peroxide according to the manufacturer's instructions) in 1:1 ethanol. After overnight incubation at 22° the tissue was further infiltrated for two 1 hr time periods with fresh, activated JB-4 solution in a Vacuum oven at 28 lb pressure and 22°. The infiltrated specimens were then placed in Beem capsules filled with activated JB-4 solution mixed with catalyst (20:1) and allowed to harden at 22°. Three micron sections were cut with a glass knife on a Porter-Blum microtome (Sorvall MT-2). The sections were stained with hematoxylin-eosin [27] or according to Wilder's method for reticulin fibers [28].

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Immunofluorescence Microscopy

Frozen specimens were placed in a Tissue Tex II cryostat (Miles Co.) and the Beem capsules were cut away. Five micron sections were cut and placed on 22 mm^2 glass coverslips. These were placed in 30-mm Falcon tissue culture dishes for subsequent handling. All incubations were carried out in 1 ml solutions at 22°. The sections were rinsed for 10 min with phosphate saline (0.15 M NaCl, 0.01 M Na phosphate, pH 7.2) containing 1% bovine serum albumin (BSA) and then for 10 min with 1% BSA, 2% goat serum in phosphate saline. They were then treated for 30 min with phosphate saline containing specific antiserum at a 1:400 dilution or normal pre-immune serum at 1:800 dilution. The sections were then rinsed 2 × 10 min with 1% BSA in phosphate saline. Next, the coverslips were incubated for 30 min with fluorescein-conjugated goat anti-rabbit antiserum. Finally, the sections were rinsed 2 × 10 min with 1% BSA in phosphate saline and the coverslips were inverted and mounted on glass slides using 0.1 M tris, 90% glycerol, pH 9.4. The sections were studied with a Zeiss Photoscope III equipped with epifluorescence.

Preparation of Specific Antiserum

Specific antiserum to guinea pig fibronectin was prepared by a method similar to that previously used to prepare specific antiserum to human plasma fibronectin [29]. Briefly, guinea pig blood was obtained by cardiac puncture and the plasma allowed to clot at 37°. Plasma fibronectin was purified by affinity chromatography on gelatin-sepharose [30] using 1M Na bromide, 20 mM Na acetate, pH 5 to elute the bound fibronectin [31]. Anti-guinea pig plasma fibronectin anti-serum was produced in rabbits [29]. The antiserum was incubated for 60 min at 37° in a 1:1 mixture with guinea pig serum from which plasma fibronectin had been removed by 2 passages through the gelatin-sepharose column. The precipitate that formed was removed by centrifugation and the resulting antiserum was tested against purified guinea pig plasma fibronectin and against whole serum. One precipitant band was observed both by Ouchterlony analysis and by immunoelectrophoresis.

Materials

Strain 13 guinea pigs were bred domestically. Sodium pentobarbitol was obtained from Abbot Labs. Innovar-vet was obtained from Pittman-Moore Co. Wescodyne was obtained from West Chemical Co. Furacin was obtained from Norwich-Eaton Pharmaceuticals. Formaldehyde (ultrapure TEM grade) was obtained from Tousimis Research Corp. Sodium cacodylate was obtained from Electron Microscopy Services. O.C.T. compound was obtained from Lab Tex Products. JB-4 methacrylate embedding kit was obtained from Polysciences. Bovine serum albumin (Fraction V) was obtained from Sigma Chem. Co. Goat serum was obtained from GIBCo. Fluorescein conjugated goat anti-rabbit antiserum was obtained from Meloy Labs. Gill-3 hematoxylin (3×) was obtained from Lerner Laboratories. Eosin Y was obtained from Polysciences Inc. Other reagent grade chemicals were obtained from Scientific Products (Mallinkrodt).

RESULTS

Sequential Events in Granulation Tissue Formation

An initial study was based on a panel of 7 guinea pigs in order to work out technical details and establish the chronology of granulation tissue formation. Subsequently, observations were made on 4 guinea pigs in rotation from the time of wounding to day 18. Frozen sections were analyzed by phase contrast microscopy and by indirect immunofluorescence after "staining" with specific anti-guinea pig plasma fibronectin antiserum to determine the distribution of fibronectin. Sections of fixed embedded material were observed following staining with H & E to characterize general cell morphology or with a silver stain to reveal reticulin fibers. (Experimental details are given in Methods and Materials.)

Stages in the organization of the granulation tissue are shown in Fig 1. It was generally easy to distinguish between the wound area and adjacent normal tissue (Fig 1*A–C*, *arrows*). There was an invasion of the fibrin clot by neutrophils and monocytes during the first 2 days (Fig 1*A*, 1*B*). By days 5 and 6, extensive neovascularization was apparent and there were numerous mononuclear cells present (Fig 1*C*, 1*D*). Foreign body giant cells were also observed (Fig 1*C*, *asterisk*). Abundant granulation tissue with fibroblasts was present by day 9 (Fig 1*E*). By day 18, extensive synthesis of connective tissue matrix had occurred and the fibroblast population was already beginning to thin out (Fig 1*F*).

Distribution of Fibronectin Prior to Wounding

The distribution of fibronectin in intact skin was similar to that reported by others [21,32]. The epidermis was negative but fibronectin was prominent at the epidermal-dermal basement membrane (Fig 2*A*, 2*B*). Fibronectin was also associated with some of the collagen fibrils at the papillary level of the dermis (Fig 2*A*, 2*B*) and to a lesser extent with collagen in the reticular level of the dermis (Fig 2*C*, 2*D*). Most of the fibrils present were judged to be type I collagen based upon their size and staining characteristics. The fibronectin positive cells in the reticular dermis were probably fibroblasts (Fig 2*C*, 2*D*). The basement membranes of the hair follical epithelium and vascular endothelium, like those of the epidermal-dermal junction, were also fibronectin-positive. Fibronectin was also found in the endomysium of the panniculus carnosus muscle (Fig 2*E*, 2*F*).

The specificity of fibronectin staining was assessed in several ways. As described in Methods and Materials, the antiserum was specific for fibronectin as determined by Ouchterlony and by immunoelectrophoretic analyses. Also, there was no fluorescent staining of skin incubated with normal, pre-immune serum (Fig 2*G*, 2*H*) or if the specific antiserum had been preincubated with excess purified fibronectin (Fig 2*I*, 2*J*).

Initial Appearance of Fibronectin in the Wound

The earliest wound analyzed was 5 hr post-operative. At this time the fibrin clot was relatively acellular. Fibronectin was associated with the clot but there was little change in its distribution in the adjacent reticular dermis (Fig 3*A*, 3*B*). Most of the fibrin strands appeared to be covered with fibronectin (Fig 3*C*). The fibrin clot did not show any fluorescent staining with pre-immune serum (Fig 3*D*, 3*E*).

Distribution of Fibronectin During the Early Phases of Granulation Tissue Development

During the first few days post-operatively, an association of fibronectin with fibrin continued to be observed (Fig 4*A*, 4*B*). There were also regions of very dense fibronectin deposition sometimes associated with thick fibrillar structures of unknown identity (Fig 4*C*, 4*D*). The presence of fibronectin was also apparent on the surfaces of the mononuclear cells (Fig 4*E*, 4*F*). Based upon the time of their appearance and observations made on H & E stained preparations, it is likely that these were macrophages. Despite the abundance of fibronectin in the forming granulation tissue, the adjacent regions of intact reticular dermis contained little of this material. When large fibrils were present within the granulation tissue, however, they often appeared to be covered with fibronectin (Fig 4*G*, 4*H*). Similar fibrils could be identified as collagen in H&E preparations and were not stained with the reticulin stain. Therefore, it is likely that they are residual type I collagen fibrils.

Distribution of Fibronectin in Mature Granulation Tissue

Most of the collagen fibrils present in well-organized granulation tissue were covered with fibronectin (Fig 5*C*, 5*D*). They were smaller than the collagen fibrils in nonwounded areas and stained with a reticulin stain (Fig 5*E*, 5*F*). These findings suggest that they are probably composed of type III collagen, which is the predominant form of collagen synthesized early in the regeneration of connective tissue matrix [33].

The day 9 specimens included some regions where epidermis had migrated over the granulation tissue. This epidermis was still fibronectin-negative, but in this case the discrete organization of fibronectin in the basement membrane was obscured by the presence of large amounts of fibronectin throughout the granulation tissue (Fig 5*A*, 5*B*; compared to Fig 2*A*, 2*B*).

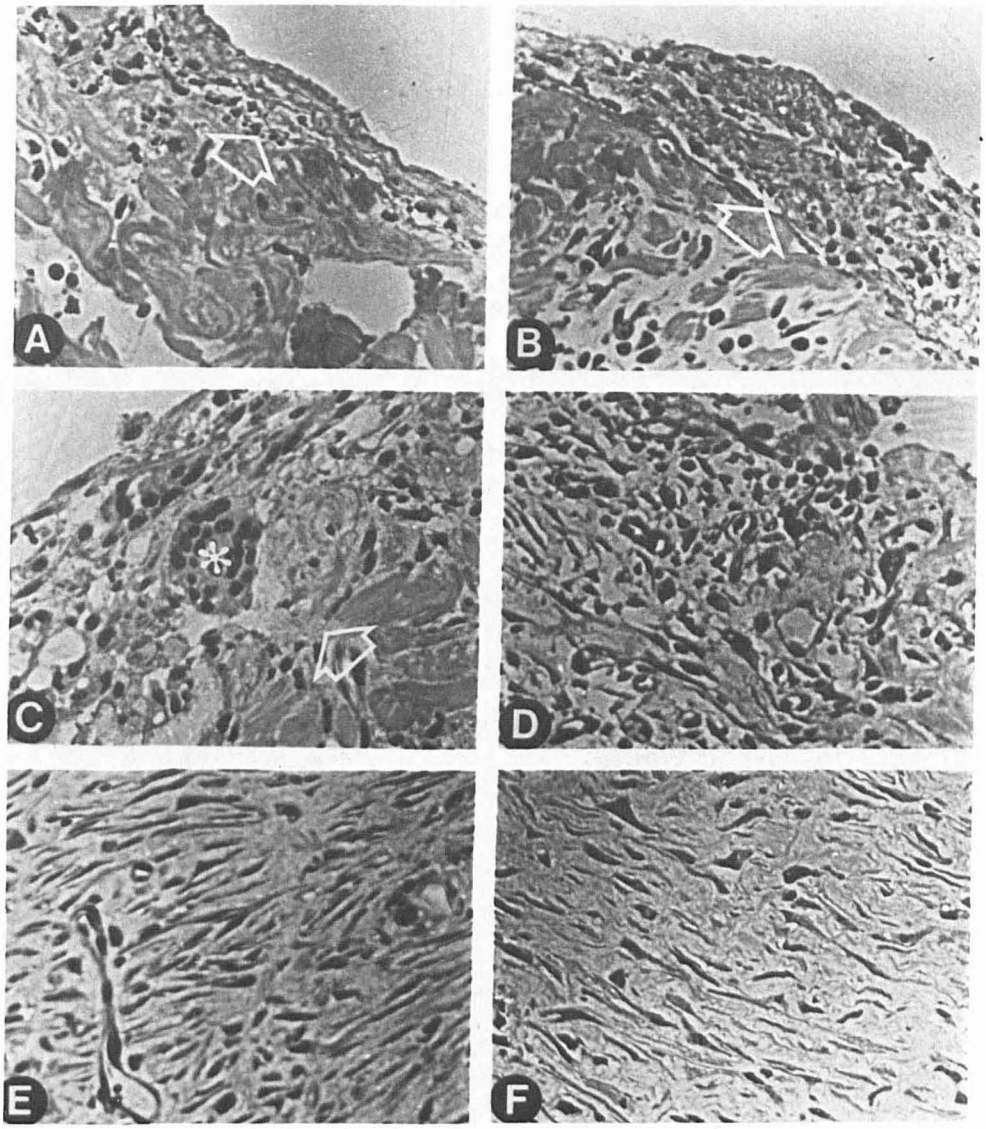

FIG 1. Appearance of granulation tissue at various times. The samples were taken as follows: Day 2 (*A*), Day 3 (*B*), Day 5 (*C*), Day 6 (*D*), Day 9 (*E*), Day 18 (*F*). The *arrows* point to the region between the granulation tissue and the unwounded reticular dermis. A multinucleate foreign body giant cell is shown by the *asterisk*. Stained by hematoxylin and eosin. Other details are in Methods and Materials (×700).

Fibronectin was still present on the newly formed collagen fibrils observed at day 14 (Fig 6*A*, 6*B*) and day 18 (Fig 6*C*, 6*D*). At day 14, cells with high surface fibronectin densities were sometimes observed in the granulation tissue (Fig 6*A*, *B*; *solid arrows*). They were generally associated with nearby capillaries (*open arrow*) and often within the vessels. Based upon the nuclear configuration of the cells observed in the frozen sections by phase contrast microscopy and in the adjacent H & E stained sections, these cells were neutrophils.

Throughout the observation period, samples were analyzed for fluorescent staining with the normal, preimmune serum as well as the specific antiserum. Little fluorescent staining was observed with the former. In the well-organized granulation tissue, however, the newly formed collagen fibrils appeared to be faintly autofluorescent (Fig 6*E*, 6*F*).

DISCUSSION

The distribution of fibronectin during healing of full thickness wounds has been studied to obtain further information about the possible role of this protein in the healing process. The findings show that fibronectin is part of the fibrin clot and subsequently this material is associated with newly deposited collagen fibrils.

The association of fibronectin with the fibrin clot is consistent with previous *in vitro* studies indicating that this material can bind to fibrin [4–6]. Most likely, the source of the fibronectin in the clot is the plasma and this may account for the decrease in plasma fibronectin levels that occurs transiently in acute inflammation or following surgical trauma and in disseminated intravascular coagulation [18–20]. It seems likely that the fibrin molecules are coated with fibronectin and therefore that fibronectin forms the substratum for subsequent cell migration. This is consistent with the requirement for fibronectin in fibroblast adhesion to fibrin *in vitro* [7]. The presence of fibronectin in the clot may also provide one of the stimuli for cell migration into the clot. This could occur by preferential adhesion if cell-fibronectin interactions were stronger than cell interactions with the surrounding matrix. Fibronectin might also promote increased migration of cells since fibroblast migration has been shown to be increased by fibronectin *in vitro* [34,35]. Significantly, fibronectin has been proposed to be part of the migratory matrix for neural crest and other embryonic cells [36,37].

Fibronectin was also observed to be closely associated with the newly deposited collagen fibrils. Based upon the size and histological staining characteristics of the fibrils, it is likely that at least in part they are composed of type III collagen (reticulin)

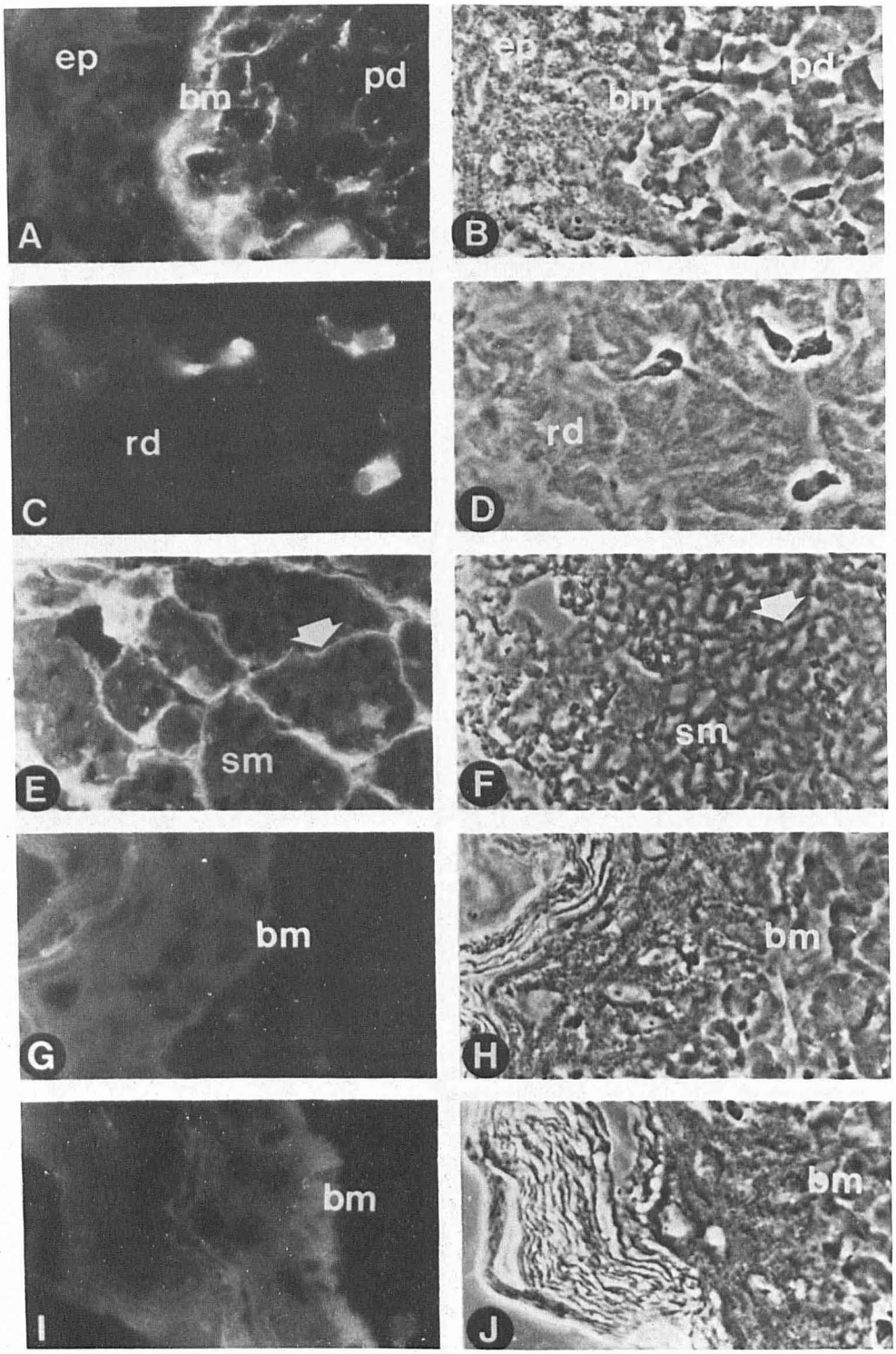

FIG 2. Fibronectin distribution in normal skin. The samples were taken at the time of wounding. *A*, *B*, *G*, *H*, *I*, and *J* show regions of epidermis (*ep*), basement membrane (*bm*) and papillary dermis (*pd*). *C* and *D* show reticular dermis (*rd*). *E* and *F* show skeletal muscle (*sm*) and the *arrow* points to endomysium. Specific antiserum was used in *A–F*. Normal, preimmune serum was used in *G* and *H*. In *I* and *J*, the specific antiserum was pretreated with purified guinea pig plasma fibronectin (0.65 mg/ml) in a 1:2 dilution for 1 hour at 37° and the mixture was then microfuged (Beckman Inst.) for 1 min. This absorbed specific antiserum was used in the immunofluorescence protocol at a dilution of 1:133. *A*, *C*, *E*, *G*, *I* are shown by fluorescence and *B*, *D*, *F*, *H*, *J* are shown by phase contrast. Other details are in Methods and Materials. (×850).

[38]. The appearance of type III collagen in the initial phases of wound healing is well established [33]. Also, the possible relationship between fibronectin distribution and reticulin fibers has been pointed out previously in studies on normal adult as well as embryonic skin [21,22]. In this regard it is important to note that *in vitro* studies with native collagens have demonstrated that fibronectin binds better to type III collagen than to any of the other types [39,40]. The source of the fibronectin associated with newly synthesized collagen in granulation tissue is unknown. One possibility is that fibronectin is part of the newly synthesized matrix. Fibroblasts in culture have been shown to secrete a combined collagen/fibronectin matrix in some studies [15–17]. On the other hand, plasma fibronectin may deposit on the new collagen fibrils after they are formed.

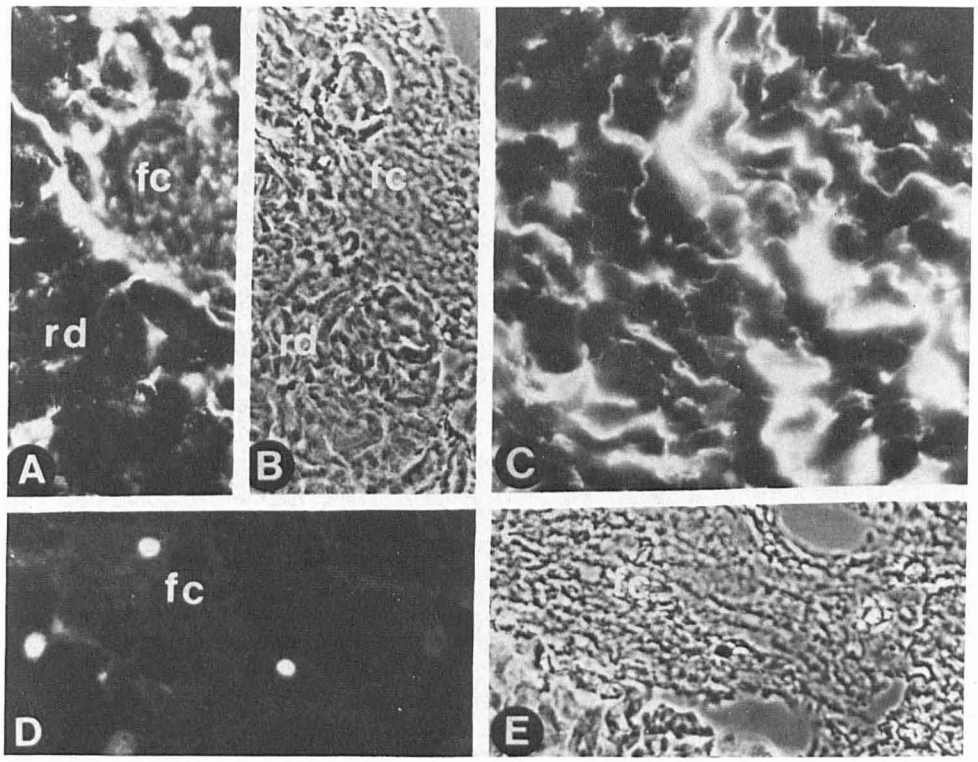

FIG 3. Fibronectin distribution in the initial clot. The samples were taken 5 hr after wounding. Regions of the fibrin clot (*fc*) and the adjacent reticular dermis are shown. Specific antiserum was used in *A–C*. Preimmune serum was used in *D* and *E*. *A*, *C*, *D* are shown by fluorescence and *B*, *E* are shown by phase contrast. Other details are in Methods and Materials (*A*, *B*, *D*, *E*, × 700; *C*, × 1075).

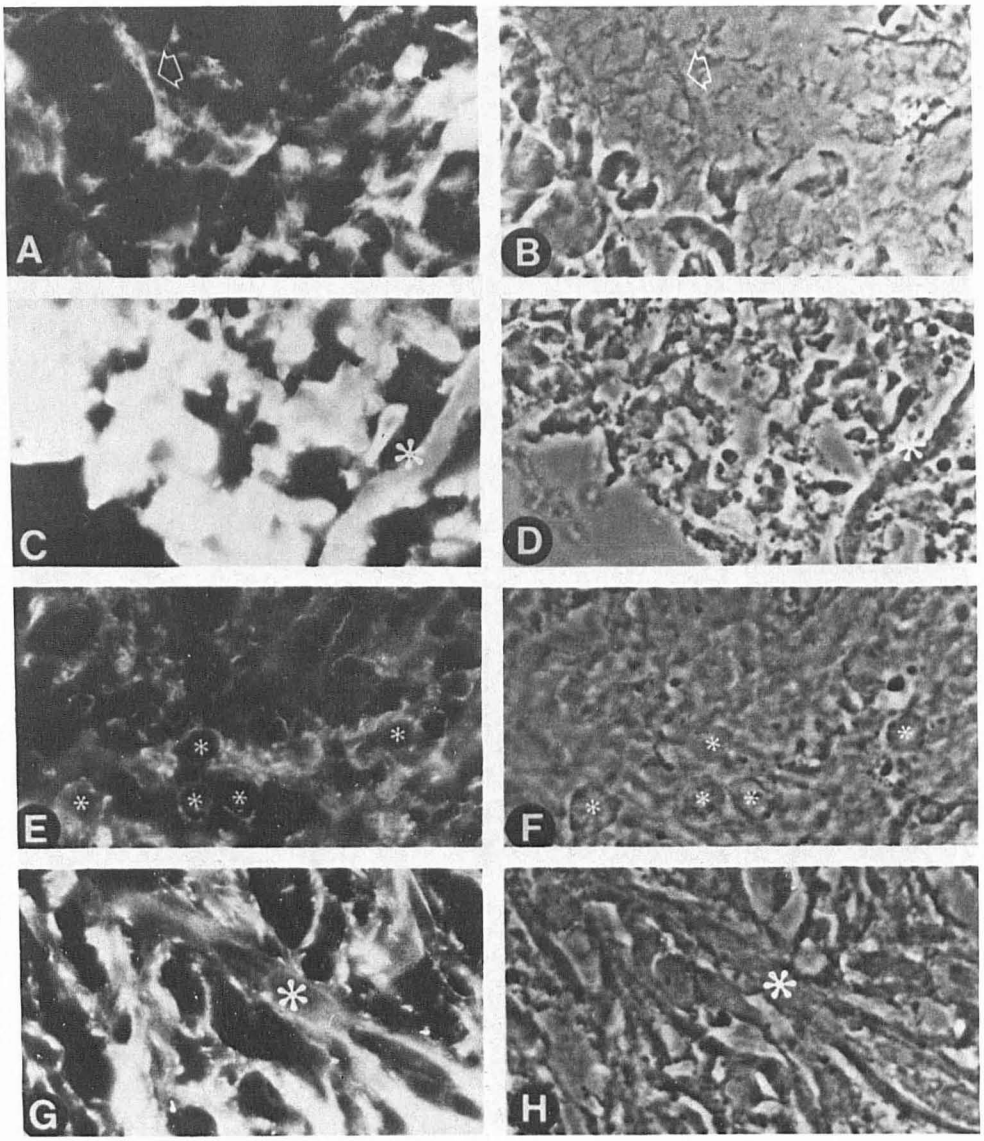

FIG 4. Fibronectin distribution in the early phases of granulation tissue organization. The samples were taken as follows: Day 2 (*A,B*), Day 3 (*C,D*), Day 5 (*E,F*), Day 6 (*G,H*). Specific antiserum was used in all of the experiments. At day 2, the *arrow* points to a fibrin strand with bound fibronectin. At day 3, the *asterisk* shows a fibrillar structure of unknown origin heavily labeled with fibronectin. At day 5, the *asterisks* show mononuclear cells coated with fibronectin. At day 6, the *asterisk* shows what is probably a type I collagen fibril coated with fibronectin within the granulation tissue. *A, C, E, G* are shown by fluorescence and *B, D, F, H* are shown by phase contrast. Other details are in Methods and Materials. (× 1050).

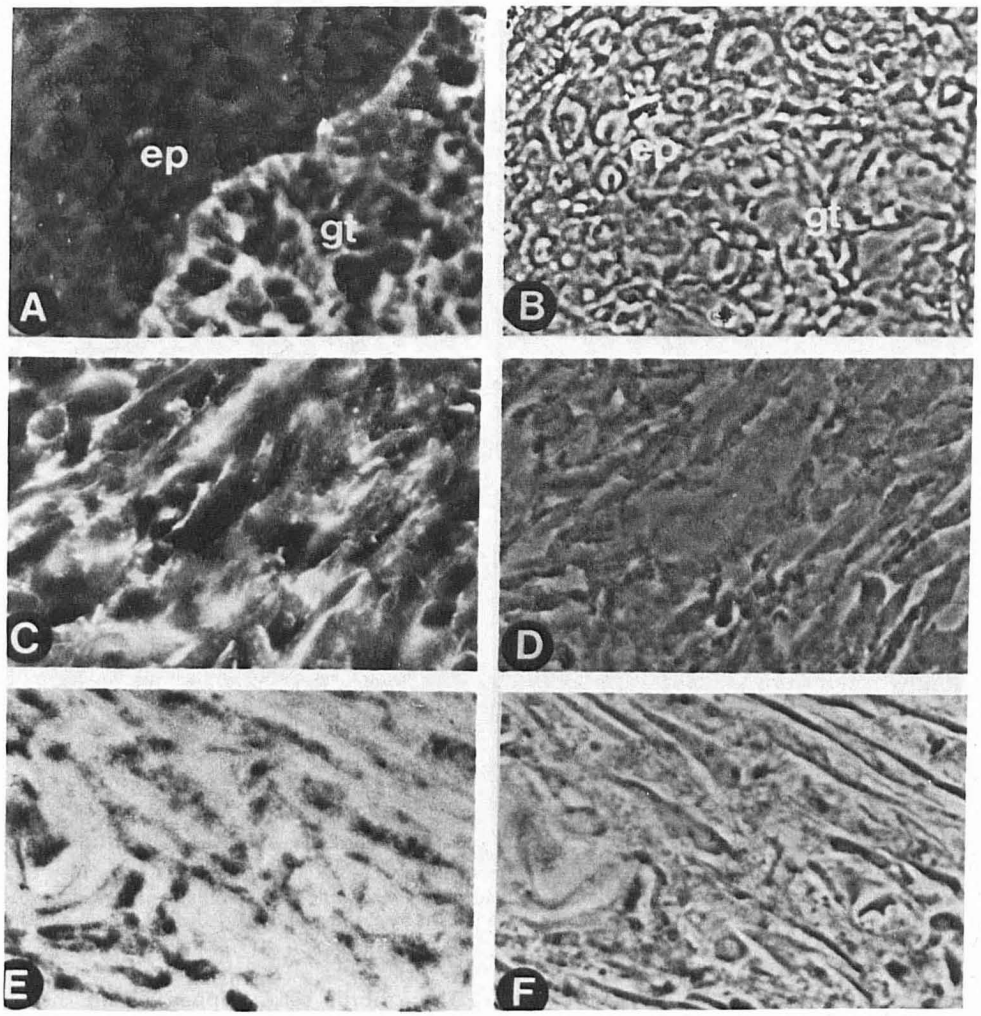

FIG 5. Fibronectin distribution in organized granulation tissue. The samples were taken at Day 9. *A*, *B* show epidermis overlying the granulation tissue (*gt*). *C–F* show granulation tissue. Specific antiserum was used in *A–D*. Silver stain for reticulin was used in *E*, *F*. *A*, *C* are shown by fluorescence; *E* is shown by light microscopy; and *B*, *D*, *F* are shown by phase contrast. Other details are in Methods and Materials (× 1075).

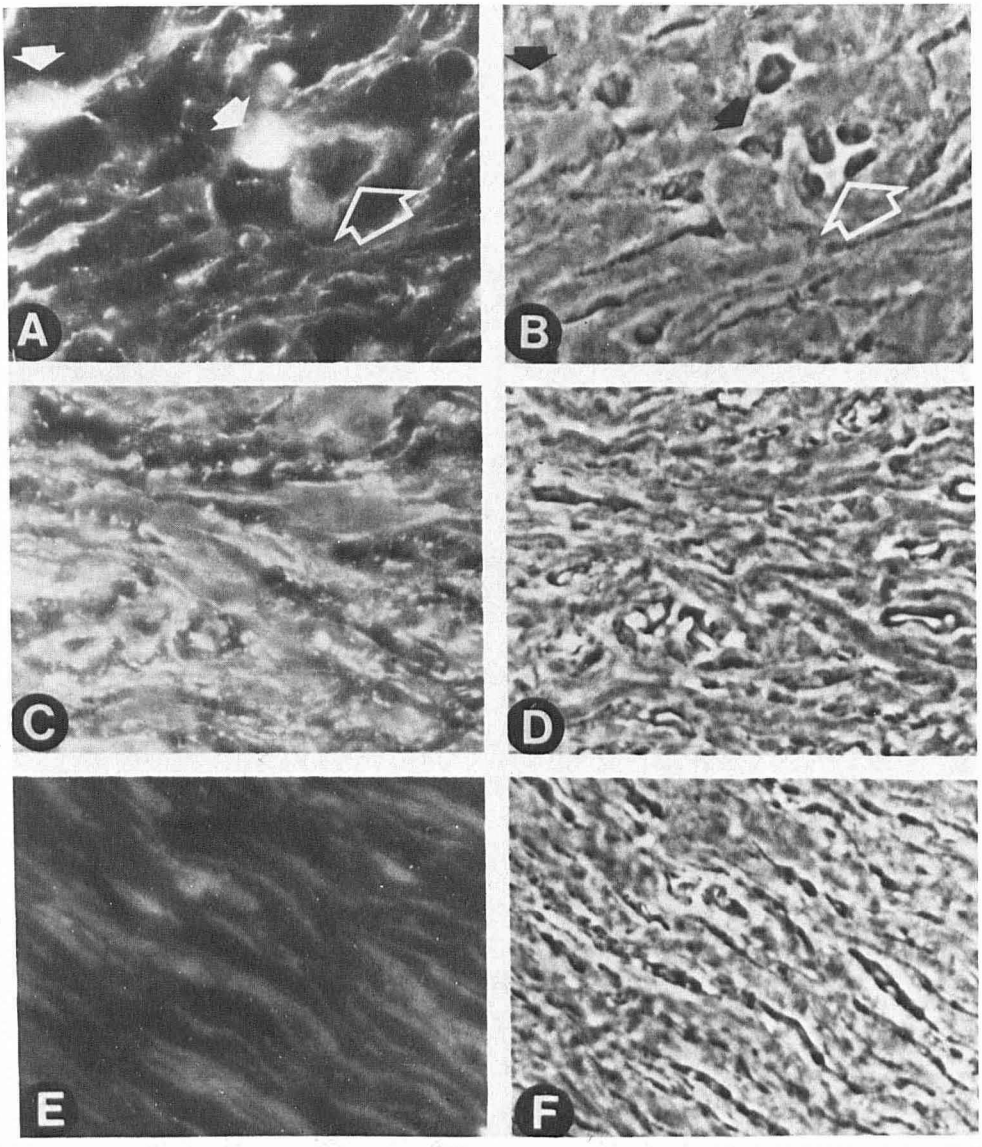

FIG 6. Fibronectin distribution in maturing granulation tissue. The samples were taken at Day 14 (*A,B*) or Day 18 (*C–F*). At Day 14 *solid arrows* point to cells heavily labeled with fibronectin which often appear to be emigrating from capillaries (*open arrow*). Specific antiserum was used in *A–D*. Normal preimmune serum was used in *E, F*. *A, C, E* are shown by fluorescence and *B, D, F* are shown by phase contrast. Other details are in Methods and Materials (×1250).

It is interesting that adjacent to the wound areas fibronectin was not associated with fibrils that had the histological appearance of type I collagen to any greater extent than was observed in the reticular dermis prior to wounding. In fact, the amount of fibronectin in normal papillary and reticular dermis is remarkably little relative to the amount observed in the granulation tissue. Also, *in vitro* studies on the adhesion of fibroblasts to native collagen gels, which maintain a three dimensional *in vivo*—like matrix [41], have demonstrated that fibronectin is not required for this fibroblast-collagen interaction [42,43]. Therefore, the role of fibronectin in fibroblast adhesion to collagen in normal dermis is unresolved.

Finally, it is worthwhile mentioning that the one time when fibronectin was observed to interact strongly with fibrils that had the histological characteristics of type I collagen was when these fibrils were trapped within the organizing granulation tissue. Since collagenase, which is prominent in wounds, causes denaturation of collagen [33] and plasma fibronectin binds strongly to all forms of denatured collagen [39,40], it is not Finally, it is worthwhile mentioning that the one time when fibronectin was observed to interact strongly with fibrils that had the histological characteristics of type I collagen was when these fibrils were trapped within the organizing granulation tissue. Since collagenase, which is prominent in wounds, causes denaturation of collagen [33] and plasma fibronectin binds strongly to all forms of denatured collagen [39,40], it is not surprising that fibronectin bound to these fibrils. Although fibroblasts are not generally considered to be highly phagocytic cells, they readily phagocytose fibronectin-coated particles [14]. In addition, fibronectin may promote the phagocytosis of denatured collagen by macrophages [12,13,44]. Therefore, the binding of fibronectin to denatured collagen fragments may be important in removal of the collagen during remodeling of wound granulation tissue.

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